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Current Opinion in Virology

Time to '*Mind the Gap*' in novel small molecule drug discovery for direct-acting antivirals for SARS-CoV-2

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A pipeline of effective direct-acting antivirals (DAAs) remains a critical gap in addressing the current pandemic given vaccination hesitancy, the emergence of viral variants of concern, susceptible populations for which vaccination is ineffective or unavailable, and the possibility that coronavirus disease 2019 (COVID-19) is here to stay. Since the start of the pandemic, global efforts in small molecule drug discovery have focused largely on testing of FDA-approved drugs to accelerate evaluation in clinical trials in hospitalized patients. With 80% of the population who test positive for SARS-CoV-2 having asymptomatic to mild COVID-19, early stage, DAAs would be of enormous benefit to reduce spread, duration of symptoms and quarantine length. We highlight a few of the most promising DAAs in clinical trials and discuss considerations in how to navigate the challenges and pitfalls of novel small molecule discovery and thereby accelerate the advancement of new, safe, and oral DAAs.

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Introduction

The global efforts to develop, test, manufacture and distribute new therapeutics and vaccines to treat coronavirus disease 2019 (COVID-19) are historically unprecedented. The successful development of several vaccines within less than a year to prevent COVID-19 represents a remarkable achievement. Since the start of 2021, communities worldwide are racing to vaccinate the human population to mitigate further waves of disease against an increase in variants of the severe acute respiratory distress syndrome-coronavirus-2 (SARS-CoV-2). Crucial gaps remain, however, in our toolbox for prevention and treatment. We face mounting challenges associated with the distribution of some vaccines requiring an advanced cold chain, vaccine hesitancy, the emergence of new variants with an ability to evade neutralization, and the possibility that this disease is here to stay. Taken together, these elements underscore a need for effective, small molecule-derived, direct-acting antivirals (DAAs) that would provide several advantages in our fight against SARS-CoV-2. First, small molecule therapeutics offer an orthogonal offensive strategy against new viral variants and for immunocompromised individuals or those who do not mount high enough levels of immunity following vaccination. Second, DAAs can mitigate outbreaks in unvaccinated populations and provide prophylactic treatment for those unvaccinated persons with high-risk exposure. Third, DAAs would be effective in mitigating spread of infection by reducing virus load in those who are SARS-CoV-2 positive and nearly or completely asymptomatic, while also reducing the length of quarantine for these individuals. Moreover, reduction of early viral load may reduce clinical manifestations of persons who have long term sequela often referred to as 'long haulers' [1•]. Lastly, DAAs offer advantages as compared to small molecules targeting host enzymes in providing action against a target not found in the host cell, potentially limiting toxicity in that regard. Hence, society needs a continued and substantial investment and shared strategies to develop DAAs to treat COVID-19 as well as the next Disease-X. The term Disease-X, coined by the WHO, represents a pathogen currently unknown to cause human disease. One such strategy is the WHO R&D Blueprint for COVID-19 which aims to fast-track the availability of effective tests, drugs and vaccines for priority pathogens by providing a roadmap and target product profiles (TPP) [2]. The major suggestion for the preferred TPP is the development of a safe, oral drug that is administered once per day and that can be rapidly scaled-up at a cost per dose that permits broad use. DAAs are urgently needed to treat the early stages of the disease where greater than 80% of infected individuals do not require hospitalization.

Antiviral drug discovery relies heavily on our understanding of both the biology and the disease caused by the pathogen of interest. The prior outbreak of SARS-CoV in China that began in 2002 [3] and the Middle East

Respiratory Syndrome (MERS) first reported in Saudi Arabia in 2012, and caused by the MERS-CoV [4], instigated global investigations into the biology and disease of these new human pathogens in the genus *Betacoronavirus* (βCoV), family *Coronaviridae*. It is from this knowledge base that the scientific community selects host or viral targets that are 'druggable' and develops robust assays to detect antiviral activity and clarify any off-target effects of the potential antiviral [5••]. Drug discovery efforts made since the emergence of these prior βCoV outbreaks [6–11] laid the foundation for a rapid, strategic therapeutic response for the COVID-19 pandemic. During 2020, most efforts in small molecule drug discovery have focused on the identification of FDA-approved drugs that might be repurposed for the treatment of COVID-19 [6,12••]. It is unlikely that additional DAA treatments will be identified from currently approved drugs given the enormous resources spent in 2020 on repurposing discovery. While this approach offered hope early in the pandemic, it is not surprising that none of these drugs has shown outstanding antiviral activity in clinical trials given that these drugs were optimized for other targets and diseases. Hence it is critical that the global scientific community supports research efforts for the discovery of novel DAAs.

A brief look at SARS-CoV-2

With a length of nearly 30 000 nucleotides, the βCoV genome is the largest of the positive-sense, single stranded RNA viruses. The genome has fourteen open reading frames that result in 16 nonstructural proteins (Nsp) and four major structural proteins, the spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins [13]. The S protein mediates cell recognition and attachment to the ACE-2 receptor, while the 3C-like cysteine protease (3CLpro or main protease, Mpro, nsp5), and papain-like protease (PLpro, nsp3) mediate the proteolytic cleavages of the polyprotein precursors, pp1a and pp1ab to produce nsp1-16. Nsp12 encodes the RNA-dependent RNA polymerase (RdRp), which along with nsp 7, 8, 9, 13 (helicase), and 14 (exonuclease, ExoN; N7-MTase) form a replicase complex (RTC) for replication and transcription of the genome [10]. Unique to the βCoV, ExoN provides a proofreading activity and corrects nucleotide misincorporation. Additional components of the viral replisome include a complex of nsp10 and nsp16 (m7GpppA capping), which methylates the cap 5′-end of the viral mRNA to enhance translation. Deletion of the SARS-CoV *nsp16* or directed mutation of the active site reduces N mRNA levels by 90% [14]. In contrast, deletion of *nsp2* shows it is not required for replication and its precise role has yet to be defined. The function of nsp11 is unknown. Nsp1 binds to the human 40S subunit in ribosomal complexes which may downregulate the immune response [15].Lastly, the nsp15 endoribonuclease (NendoU) functions to suppress interferon activation [16].

Before the COVID-19 pandemic, a number of labs reported novel and repurposed DAA molecules that inhibit entry or replication of SARS-CoV and/or MERS-CoV [5••,11]; for examples, see the S protein [17], the Mpro [18], the papain-like cysteine protease, PLpro, [19], the RdRp [20] and N-7 MTase [21]. This prior research provides a strong premise for the notion that effective DAAs to treat COVID-19 can be discovered and developed. Of these viral proteins, the RdRp and proteases are highly attractive therapeutic targets [9] as demonstrated by existing FDA-approved antiviral therapies for human immune deficiency virus and hepatitis C virus [22]. The Nsp15, an endoribonuclease, endoU, is not essential for replication, but is critical for evasion of cytoplasmic pattern recognition receptors (PRRs), and hence in contrast to an RTC inhibitor, targeting this activity would attenuate viral-induced pathogenesis [23]. Ideally, combination therapy could be developed for DAAs attacking the RTC, protease, and the IFN antagonism mediated by the Nsp15.

Direct-Acting antivirals under evaluation for treatment of COVID-19

As of May 2021, only Gilead's remdesivir (GS-5734), an IV injectable nucleoside analog that broadly targets viral RNA polymerases, has been given an emergency use authorization by the FDA for use in treatment of COVID-19 as it shortens patient recovery time; though patients receiving this treatment still suffer a high mortality rate [24•]. While IV administration is compatible for treatment of hospitalized patients, this requirement presents a significant challenge in treatment of exposures (prophylactic), asymptomatic or mild symptoms, as these individuals will not be admitted to overburdened hospitals and would benefit from an orally bioavailable drug that can be obtained from the local pharmacy. Repurposed, broad spectrum, nucleotide analog antivirals targeting replication that have advanced into clinical trials include favipiravir [25], MK-4482 (molnupiravir, EIDD-2801) and AT-527 [26]. Favipiravir inhibits by a combination of chain termination, slowed viral RNA synthesis, and lethal mutagenesis, while MK-4482 is a cytidine nucleoside analog and acts as a mutagen like ribavirin [27]. While *in vitro* antiviral activity of favipiravir was modest, high doses reduce virus load in Syrian hamsters infected with a high dose of SARS CoV-2 [28,29]. Promising efficacy of MK-4482 against the SARS-CoV-2 has been demonstrated in the hamster [30] and ferret [31]. As discussed by Kaptein *et al.* and recently [29], it is unknown if such a high dose of favipiravir may be used safely in humans, and therefore, its use may be limited in terms of its exposure, tolerance, and toxicity. While no animal studies have yet been reported, the *in vitro* activity of the guanosine nucleoside analog, AT-527, is approximately 8-fold lower than MK-4482 using normal human airway epithelial cells [26]. In mid-April, Merck announced that MK-4482 did not demonstrate a clinical

benefit in hospitalized patients so they are proceeding with a Phase 3 trial program in non-hospitalized patients.

Inhibitors of SARS-CoV-2 proteases have been highly sought from the start of the pandemic. Pfizer has started phase 1 trials on a covalent, reversible DAA, PF-07321332, that targets the 3CLpro. The drug can be administered orally. The advantage of a covalent, reversible inhibitor is tremendous, as the covalent binding will increase the residency time of an inhibitor at the site of action and reduce the dose needed for efficacy, thereby potentially widening the therapeutic index. Humans do not have a comparable 3CLpro homologue suggesting a high selectivity of PF-07321332.

Accelerating the hit to lead to preclinical path

Despite the considerable loss of human life attributable to viral infections worldwide, there have only been roughly 100 FDA-approved antiviral drugs developed in about half as many years [22]. As appreciated by others [32•], each step from basic discovery to preclinical evaluation requires adequate planning, establishment of criteria and a roadmap of milestones to accelerate the timeline. Moreover, this planning must take into consideration the biology of the virus which may have additional challenges not common in other diseases. For example, RNA viruses have a high genome plasticity due to the high error of the misincorporation of nucleosides by the RdRP during replication, which results in a high mutation frequency. As a consequence, selective pressures from DAA treatments may lead to resistance, or even enhance virus replication [33] or persistence [34]. Thus, an RNA virus antiviral campaign should evaluate resistance from the start of the program. The evaluation of resistance will not guarantee success, but to not do so increases the chance of failure. We discuss multiple procedural elements which may aid those who are new to the antiviral discovery effort for COVID-19. Attention paid to these components may accelerate the discovery pipeline and mitigate common pitfalls. Herein, we focus on four key considerations; validation of the target as druggable, establishment of a roadmap from hit to lead, translation of the efficacy data from *in vitro* to *in vivo*, and target exposure considerations.

Substantiate the target's validity in the therapeutic window

The study of disease progression in case studies provides important insight into the optimal therapeutic window during which target engagement of the protein or enzyme may impact the course of the infection and resulting disease. It is critical, then, if one aims to develop an antiviral drug against a specific viral protein that the viral target is playing a critical role in infection and/or in disease progression when patients are tested or enter clinical care. For the SARS-CoV-2, we know that once an individual is infected that symptoms may show within 12 days on average, and the virus load increases in the respiratory tract 2–3 days before symptoms appear with the peak titers being reached at symptom onset [35]. For most persons, the virus load declines following symptom onset for about 7–8 days although in worse COVID-19 cases this may be longer. After a period of >9 days, for persons showing persistence of viral infection, this does not necessarily equate to shedding of infectious virus [36]. For asymptomatic individuals who are identified through contact screening, one would expect a similar timeline for infection. Hence this information lends strong support to the benefit a DAA would provide in terms of reduction of virus load before 9–12 days post-infection. Current controlled, clinical trials to test the efficacy of favipiravir and/or lopinavir/ritonavir are enrolling subjectswithin the first 5–7 days of symptom onset or within 48 hours of a positive SARS-CoV-2 test [37]. Remdesivir did lessen the length of stay in the hospital when given early in symptom onset, similar to oseltamivir [38].

Begin with the 'hit' criteria defined

In addition to a thorough evaluation of the target, fueling the drug discovery pipeline with high quality antiviral compound hits that can be developed into promising clinical candidates pivots critically on early stage decisions. First, the minimal characteristics of a hit compound should be defined. Hits that exhibit EC_{50} values $\leq 10\ \mu M$ are commonly selected as starting points due to consideration of adequate target affinity, cell permeability, solubility and resources required to optimize these early hits into compounds that are more suitable for anticipated *in vivo* work. Though tempting, prioritizing compounds based on potency alone ignores other crucial aspects such as efficacy, toxicity, selectivity, promiscuity such as frequent hitters or PAINS [39], solubility and synthetic feasibility. A global assessment of these factors and others may dramatically alter which compounds are prioritized. As such, the most potent hit compound may not be the most promising when all points are considered. Second, validated assays that provide reliable and timely data should be established to advance compounds based on these variables [40]. Biochemical and functional assays, along with an assessment of cytotoxicity and selectivity, are commonly employed, as are orthogonal assays with different readouts from the primary assays that help identify and triage false positives. Taking a holistic approach to the dataset, which includes a thorough evaluation of the shape of the dose response curves rather than relying on EC_{50} values determined from them, mitigates some risk of failure or false starts. Third, the initial hits need to be vetted in terms of structural integrity and purity before being resubmitted for confirmation in the primary assays. Implementing these practices can reveal issues such as a misassigned structure, a decomposed sample, or an artifact of the assay for a given hit compound. Notably, trace metals left behind from a

synthesis are undetectable by the traditional spectroscopic or analytical methods used to assess structure and purity, and they can generate misleading activity in antimicrobial assays as many pathogens are sensitive to the contaminating metals. Literature and patent reconnaissance of a hit scaffold will also highlight known synthetic issues, relevant pharmacology, off-target liabilities, and intellectual property position, any one of which may influence hit selection.

High quality hit compounds share general characteristics (Figure 1). Superior hits not only meet these collective standards but also show structure-activity relationships (SAR) among structural analogs that suggest that multiparameter optimization will be permissible. If insufficient breadth of SAR is apparent, the series should likely be dropped in favor of a better performing hit scaffold from the 2–3 that are typically explored in parallel. While seemingly counterintuitive, a goal of early hit evaluation and hit-to-lead activities involves eliminating a hit series as soon as possible with the fewest possible analogs. Prolonging efforts on an inferior structural series drains precious resources and time away from the overall goal. This process provides a strong foundation from which prized hits can be identified and improved analogs can be reasonably designed. Promising compounds are positioned as part of a more robust optimization effort that is supported with tiered ADME assessments, parsed with clear gating criteria to advance those compounds with the essential attributes into more discriminating evaluations leading to determination of *in vivo* pharmacokinetic (PK) parameters in a relevant animal model. Optimization of ADME and PK with iterative checks on potency, selectivity and toxicity forge a path to examining *in vivo* efficacy.

Potential challenges in the translation of *in vitro* antiviral activity into *in vivo* antiviral efficacy

Once one identifies an early lead, one must translate its activity through a series of preclinical *in vivo* studies to establish a framework for Go/No-Go decisions. In this phase, one will evaluate *in vivo* efficacy, pharmacokinetics in select preclinical species (rat, dog), as well as tolerability, cytotoxicity and off-target toxicity.

Multiple challenges may complicate the translation from *in vitro* to *in vivo* efficacy. One such factor is off target protein binding. Most screening assays for antiviral drug candidates are either based on isolated protein interaction assays to determine the concentration-effect relationship of binding and/or inhibiting isolated viral protein targets, or on cell-line based assays that assess some level of viral inhibition in a mammalian host cell system. Such assays are widely used in high throughput assays to screen chemical compounds for their activity against the target or virus of choice. These assays are usually performed in protein-free (or low protein-containing) media. In contrast, in the *in vivo* environment in plasma and interstitial fluids, the aqueous environment is rich of plasma and tissue proteins. Because of their commonly high lipophilicity, many compounds with antiviral activity in *in vitro* assays exhibit substantial binding to plasma and tissue proteins (e.g. 88–94% of remdesivir binds human plasma proteins, but only 1–2% after its active metabolites have been formed intracellularly [38]). It is a general pharmacological principle that only the free, unbound fraction of a compound can be taken up intracellularly, can interact with its target structure and has pharmacologic activity [41]. Therefore, protein binding oftentimes substantially limits the potency of compounds of interest *in vivo*. A protein binding of 90% will shift the IC_{50} of a candidate

Figure 1

Primary hits

- Show favorable physiochemical properties (molecular weight and Log D)
- Lack reactive or unstable chemical functionality
- Have confirmed structure and purity (≥ 95% by UV detection)
- Avoid known frequent hitter motifs (PAINS)
- Show low promiscuity (<5% across diverse targets)
- Provide reproducible and correlative data from primary and secondary assays
- Demonstrate a reasonable activity profile (EC_{50} ≤ 10 μM, selectivity ≥ 5-fold)
- Exhibit low cytotoxicity (CC_{50} ≥ 10-fold EC_{50})
- Produce reproducible, high quality dose response curves
- Are synthetically feasible (short, highly diversifiable sequences preferred)

Advanced hits

- Show solubility within range of the assay IC_{50}/EC_{50} values
- Supported by inherent or scouting SAR
- Associated with a clear path forward in items of intellectual property (IP)

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Ideal attributes of validated hit compounds.

compound as a measure of potency from 0.1 μM in an *in vitro* assay to 1 μM *in vivo*. Thus, overly optimistic outlooks based on *in vitro* potencies are often diminished by protein binding effects into therapeutically insufficient potencies *in vivo*. These considerations are further complicated by the fact that different animal species oftentimes have different degrees of protein binding, usually captured as plasma protein binding. Thus, careful assessment of binding differences between humans and different preclinical animal species used in the evaluation of drug candidates needs to be considered to allow for a meaningful extrapolation of drug potencies from *in vitro* settings to preclinical animal models, and ultimately to humans.

How to correctly assess target exposure necessary for therapeutic efficacy *in vivo*

Drug exposure over time in target tissues where the viral infection resides is the main driver for therapeutic efficacy. Thus, to determine whether a specific dosing regimen is likely to be effective, it is prudent to determine the pharmacologically active concentrations that result from this dosing regimen in target tissues. Unfortunately, tissue drug concentration assessments are often performed as tissue homogenate assessments, rather than determination of therapeutically relevant free concentrations, as for example performed by microdialysis techniques [42]. Tissue homogenate concentrations used in lieu of real pharmacologically active concentrations become especially problematic if the drug is sequestered in cellular or subcellular structures that do not contribute to anti-infective activity.

While not a DAA, the EUA for hydroxychloroquine (HCQ) for COVID-19, and its subsequent withdrawal less than three months later, provides a prime example for the fallacy to ignore basic pharmacological principles of drug distribution in the extrapolation of *in vitro* activity to *in vivo* efficacy. Initial enthusiasm for the potential efficacy of HCQ against SARS-CoV-2 *in vivo* was based on a low antiviral EC_{50} in *in vitro* assays and its substantially higher concentrations in lung homogenates relative to plasma. In a physiological pharmacokinetic modeling approach, Yao *et al.* used this lung-to-plasma partition coefficient to predict that the free HCQ trough concentrations in lung were expected to be 21-fold to 169-fold higher than the *in vitro* derived EC_{50} value with dosing regimens known to be tolerated in humans [43]. The high HCQ tissue concentrations, however, are the consequence of extensive sequestration of HCQ in acidic intracellular organelles such as endosomes, Golgi apparatus and lysosomes. These high concentrations in select intracellular structures are not reflective of the HCQ concentrations in the cell culture media used for *in vitro* potency assessments; rather, the free drug concentration in the interstitial space of the lung, which is generally assumed to be for most drugs equivalent to the free, unbound drug concentration in plasma should be used to translate *in vitro* antiviral activity to drug exposures and corresponding dosing regimens required for *in vivo* activity. Following this line of thought, FDA staff scientists convincingly showed that antiviral activity against SARS-CoV-2 is not likely achievable with safe HCQ dosing regimens in humans [44••], leading together with disappointing clinical results to the EUA revocation. Ou *et al.* [45] provides additional scientific factors why HCQ might have been ineffective *in vivo*. This example underlines the importance to consider drug distribution processes when *in vitro* potency measures are used to predict *in vivo* exposure and dose requirements.

Conclusions

On the precipice of almost a year and a half into the COVID-19 pandemic, there remains a global need for antivirals that reduce viral spread, disease severity and death. The continual emergence of new SARS-CoV-2 variants underscores the need for small molecule therapeutics to treat this viral infection and disease [46] beyond the influence of vaccines. To reach that goal, we must learn from and invest in the lessons learned from past viral outbreaks which may be the harbingers of the next Disease-X. Further, acute infectious diseases must once again become a priority for drug discovery, and development efforts need to consistently apply rigorous rubrics for the hit-to-lead and lead optimization processes.

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Conflict of interest statement

Nothing declared.

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